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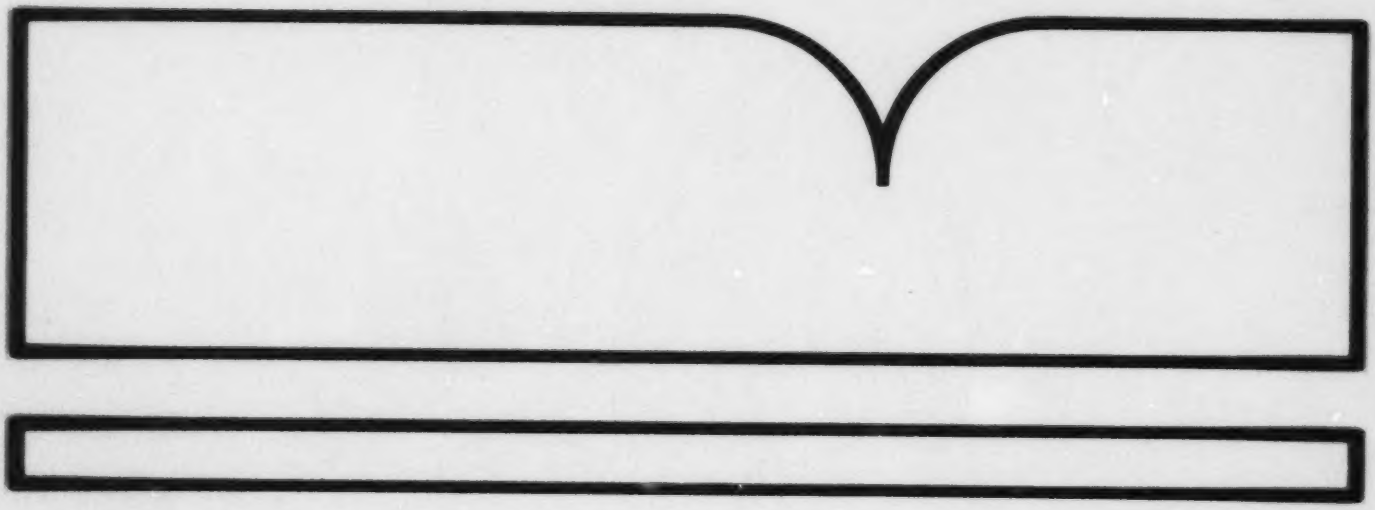

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16. ABSTRACT

A committee of scientists was established by the U.S. Environmental Protection Agency to appraise the current state of aneuploidy test methodology, to compile and analyze published data on the chemical induction of aneuploidy, and to provide guidance for additional test development and validation. The committee was organized into 9 working groups, representing different eukaryotic organisms ranging from fungi to whole mammals. The Environmental Mutagen, Carcinogen, and Teratogen Information Program at Oak Ridge National Laboratory identified and provided literature to the working groups. The 9 reports were prepared by the individual working groups. The reports include: (1) descriptions of the available testing approaches and general principles to be followed for protocol development, (2) criteria for evaluating data, and (3) recommendations for future test development and basic research. The evaluated results are presented in tables. For the positive and negative calls, the lowest effective dose tested or the highest ineffective dose tested, respectively, and the range of concentrations tested are provided. An overall summary report was prepared that summarizes the conclusions and recommendations of the 9 working groups and the chemical data base. It is hoped that these reports will provide a framework for further test development and validation of aneuploidy assays.

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Aneuploidy in mammalian somatic cells in vivo *

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* Report of the Aneuploidy Data Review Committee (Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC).

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Summary

Aneuploidy is an important potential source of human disease and of reproductive failure. Nevertheless, the ability of chemical agents to induce aneuploidy has been investigated only sporadically in intact (whole-animal) mammalian systems. A search of the available literature from the EMCT Aneuploidy File (for years 1970–1983) provided 112 papers that dealt with aneuploidy in mammalian somatic cells in vivo. 59 of these papers did not meet minimal criteria for analysis and were rejected from subsequent review. Of the remaining 53 papers that dealt with aneuploidy induction by chemical agents in mammalian somatic cells in vivo, only 3 (6%) contained data that were considered to be supported conclusively by adequate study designs, execution, and reporting. These 3 papers dealt with 2 chemicals, one of which, mercury, was negative for aneuploidy induction in humans, and the other, pyrimethamine, was positive in an experimental rodent study. The majority of papers (94%) were considered inconclusive for a variety of reasons. The most common reasons for calling a study inconclusive were (a) combining data on hyperploidy with those on hypoploidy and/or polyploidy, (b) an inadequate or unspecified number of animals and/or cells per animal scored per treatment group, and (c) poor data presentation such that animal-to-animal variability could not be assessed. Suggestions for protocol development are made, and the future directions of research into aneuploidy induction are discussed.

(1) Introduction

Although aneuploidy has been recognized for many years as a serious danger to human health, the induction of aneuploidy by chemical agents has received little attention from the perspective of population health and environmental impact when compared with the attention given to gene and chromosomal mutations. Assays to assess the ability of chemicals and other agents to induce aneuploidy have existed for many years, but most of these test systems use submammalian species. Mavor (1921; cited in Bond and Chandley, 1983) first reported the experimental induction of aneuploidy by X-irradiation in *Drosophila melanogaster*. Studies on aneuploidy induction by experimental or therapeutic regimes in mammals or humans have been slow to follow. In 1968, Ferreira and Buoniconti reported 'a significant increase of cells with abnormal numbers of chromosomes', hyperploidy as well as polyploidy, compared with control levels in the lymphocytes of human patients treated with anti-gout colchicine therapy. In an experimental in vivo mammalian study, Colnaghi et al. (1969) reported increased aneuploidy in the thymuses of CTM and C3Hf mice exposed intraperitoneally to urethane. Subsequent studies on aneuploidy induction in mammalian in vivo systems have been relatively few. The majority of the aneuploidy data presented from mammalian studies has been obtained as ancillary numerical observations in clastogenic studies and therefore often suffers from inadequacies of procedure, observation, reporting or interpretation. To determine what data are currently available on the aneuploidy-inducing capability of chemical agents in somatic mammalian cells in vivo, it was appropriate to review the

existing literature and to assess the validity of the published data.

The Aneuploidy Data Review Committee working group on mammalian somatic cells in vivo evaluated 112 papers, dated 1970–1983, provided by the Environmental Mutagen, Carcinogen and Teratogen Information Department (EMCT), Oak Ridge National Laboratory. 59 of this initial group of papers were rejected from subsequent data evaluation by criteria established by the Review Committee (Dellarco et al., 1985). The papers in each rejection category were as follows: (a) 13 papers lacked original data; (b) 13 papers presented data which did not directly reflect aneuploidy: (c) 6 papers lacked negative control data; (d) 19 papers dealt with agents that were not chemical; (e) in 5 papers, the test system was exposed simultaneously to more than one agent; and (f) 3 papers dealt with ill-defined agents or systems (i.e., 'oral contraceptive', 'adenocarcinoma-bearing mice').

Papers with micronucleus induction as endpoint were not reviewed. Although it is recognized that nondisjunction is a mechanism that can lead to micronucleus formation, these micronuclei cannot be distinguished unequivocally from those produced by clastogenic action (e.g., acentric fragments: Heddle et al., 1983). One paper dealing with micronucleus data (Seiler, 1976) was retained because it also included data on anaphase effects and structural aberrations which, in combination with the micronucleus data, in theory may allow an assessment of aneuploidy induction (Section 2.7).

3 papers dealing with related endpoints (centromere separation, C-mitosis, diplochromosomes, nuclear DNA content) were considered by this working group as 'not aneuploidy', and rejected from further review. These and other endpoints may prove useful as directions for future assay development (Section 6).

(2) Specific considerations used in the detailed evaluation of papers

In the detailed evaluation of the 53 papers remaining after the initial rejections, most results were judged by the working group to be inconclusive, in most cases notwithstanding a definite call of positive or negative by the author(s). Any one of the following deficiencies generally was sufficient to warrant that the data presented in the paper be considered inconclusive.

(2.1) Hypoploidy or hyperploidy data reported only as a percentage without indication of the numbers of cells scored

The absolute number of hypoploid or hyperploid cells could not be reconstructed, and no allowance could be made for the significance, if any, of different sample sizes.

(2.2) Only aneuploidy reported, with no separation into hypoploid and hyperploid components

For studies with an apparently increased frequency of aneuploidy, it was not possible to determine whether the incidence of hyperploids was increased. An increase in hypoploidy alone might reflect technical artifacts, or an agent-induced increase in cell fragility. Such data do not constitute sufficient evidence of true aneuploidy induction. For studies in which the frequency of aneuploidy was *not* significantly increased, an increase in hyperploidy might be masked by the larger frequency of hypoploidy with which it is combined.

(2.3) Hyperploidy and polyploidy reported as a combined figure

This does not permit the distinction of aneuploidy- from polyploidy-inducing agents. Although some agents may induce both endpoints, there is insufficient evidence at present to conclude that this is always the case. Furthermore, polyploid cells occur normally in some tissues (e.g., bone marrow), and an increased frequency of polyploid cells in these tissues may not be related to aneuploidy induction. Finally, the combining of polyploid and hyperploid data may mask alterations in hyperploid frequencies.

(2.4) The number of animals and / or cells per treatment group too few or not specified

In treatment groups comprised of too few animals, one of the main components of experimental variability, animal-to-animal differences, cannot be ascertained adequately. If unequal numbers of cells are sampled from different animals

within a treatment group, the results may be biased toward an atypical response. In in vivo animal studies, the sample size is the number of animals. For statistical analysis, the evaluated parameter is frequency per animal. While the appropriate number of animals to be used cannot be determined at the present time, a very small number (e.g., 1 or 2) would not allow for statistical analysis using animal-to-animal variability.

(2.5) Accurate dose information not given for experimental exposures to chemicals purportedly negative for aneuploidy induction

An example of inexact dosage is provided by a study on carbon disulfide, in which the dose level is given only as 1/10 to 1/100 of the LD_{50}, but the value of the LD_{50} is not provided.

(2.6) Incomplete treatment protocol: one dose level and one treatment time, with a negative response

Although an experiment with only one dose level tested or one sample time may produce positive evidence for aneuploidy induction, a negative response under the same conditions may not be valid. Without information on the dose–response curve for a chemical, a single dose level risks being either too low or too high to induce aneuploidy. A single sample time, depending on the extent of proliferative delay induced by the chemical, risks being either too early or too late for scoring of aneuploid cells (Section 2.7).

(2.7) Inappropriate treatment protocol: sampling

Within continuously proliferating cell populations, aneuploid cells may be lost over time through cell death or through diminished growth potential; sampling such a population at extended times after administration of a nonpersistent compound may underestimate the extent of aneuploid induction. A short sample time that does not allow for an intervening mitosis would not permit the induction of aneuploid cells. 1- and 3-h post-exposure harvests used in an evaluation of ethanol are examples of the inappropriately early sampling of cells that could lead to false negative calls. Until more is understood about the kinetics of aneuploidy expression, a range of several sacrifice times should be included in the study design. Another example of improper sampling was a study (Seiler, 1976) that did not report numerical counts, but 3 other endpoints (micronucleus induction, anaphase effects and structural aberrations). These endpoints, in combination, could indicate aneuploidy induction, except for the fact that, in this particular study, 2 different species were used for the 3 different endpoints. The possibility of species differences in metabolism or sensitivity could not be excluded, and the study was termed inconclusive.

(2.8) Unacceptable background frequency

An excessively high background frequency may indicate difficulties in performance of the assay, improper quality control in slide preparation, or scoring errors. At present, it is difficult to establish the acceptable range of background frequency for aneuploidy because of the limited data base. A frequency of hyperploidy in excess of about 2% probably is due to artifacts of study execution. The subject of baseline frequencies is discussed in more detail in Section 5.

(2.9) Positive response for hypoploidy and negative response for hyperploidy

Because mechanisms other than chemical induction may produce hypoploid cells (Section 2.2), a positive hypoploidy/negative hyperploidy score cannot be considered as positive evidence of aneuploidy induction. Similar reasoning applies to polyploidy (Section 2.3).

(2.10) Data not presented in such a manner as to allow assessment of animal-to-animal variability

Inability to reconstruct the numbers of animals per treatment group or the numbers of cells per animal precludes statistical evaluation of the significance of an effect.

The extremely poor yield of conclusive results from the current literature review is disappointing. It reveals the inadequate development of this endpoint and indicates the need for a more rigorous approach to study design and reporting. Some factors to be considered in developing such data in the future are given below (Section 4).

(3) Evaluation of the literature

Of the 53 papers that passed the initial series of rejection criteria, only three contained sufficient

information to allow a definite judgment on the accuracy of its assessment of aneuploidy induction. Evaluations were classified as positive or negative. A chemical was considered positive for aneuploidy induction if the frequency of hyperploid cells in the exposed animals or humans was significantly elevated above the control levels by the statistical analysis of the original authors, and when the statistical method used was not judged inappropriate or seriously deficient. A chemical was considered negative for aneuploidy induction if, by statistical analysis of the original authors, there was no significant increase in hyperploid cell frequency compared with the control levels. A third category, inconclusive, was used for the 50 papers in which study design or data presentation failed to meet the minimal criteria specified in Section 2.

In Tables 1 and 2, the chemical name, CAS registry number, species, tissue(s), reference and reason(s) for a call of inconclusive are provided. In Table 1, the highest ineffective dose or lowest effective dose tested (HIDT or LEDT, respectively), dose range tested, and decision of the working group are also included. In Table 2, the decision of the working group is inconclusive for all listed chemicals.

Studies on 2 chemicals, mercury and pyrimethamine, contain data that permitted a decisive call of positive or negative, as presented in Table 1. This table refers to an additional paper on mercury which, although inconclusive, is included here in order to kept all data for a given chemical together.

Mercury was negative for aneuploidy induction in human peripheral blood lymphocytes in two studies from occupational or environmental exposures. Some of the stringency expected of experimental studies was not required of human studies: precise dose information and appropriate sampling times could not be demanded of human exposures because prospective dosing is not always possible. In cases of human occupational or environmental exposure, the exposure levels usually can be estimated only. These negative results must be viewed with some caution, because higher exposures to mercury may reveal aneuploidy-inducing capability not apparent at the lower exposure levels. Verification of the negative results obtained in humans by testing metallic mercury in an experimental rodent in vivo system is highly recommended.

Pyrimethamine (chloridin, an antimalarial drug) was positive for aneuploidy induction in male rats exposed per os to 50 mg/kg body weight, the only dose tested. Bone marrow cells were examined 48 h after an acute exposure to the drug.

63 chemicals from 51 papers have data only

TABLE 1

RESULTS FROM PANEL REVIEW: WHOLE MAMMAL SOMATIC CELLS

Chemical	Mercury (metallic)	Pyrimethamine
CAS Registry No.	7439-97-6	58-14-0
Species	Man	Rat
Tissue	Peripheral blood lymphocyte	Bone marrow
HIDT [a]	Occupational	
LEDT [b]		50 mg/kg
Dose range	Occupational	50 mg/kg, plus control
Panel call [c]	−; −; I	+
Reference(s)	Verschaeve et al., 1976, 1979b; Popescu et al., 1979	Barilyak et al., 1978
Primary reason(s) [d] if inconclusive	NA; NA; 2, 3	

[a] Highest ineffective dose tested.
[b] Lowest effective dose tested.
[c] +, positive; −, negative; I, inconclusive.
[d] Numbers correspond to reasons given in Section 2.

TABLE 2

DATA ON STUDIES DEEMED INCONCLUSIVE UPON PANEL REVIEW

Chemical (CAS Registry No.)	Species [a]	Tissue [b]	Reference	Primary reason(s) for inconclusive [c]
2-Acetylaminofluorene (53-96-3)	rat	liver	Masashito et al., 1976	4
Acrylamide (79-06-1)	mouse	BM	Shiraishi, 1978	3
Aflatoxin B_1 (1162-65-8)	rat	liver	Masashito et al., 1976	4
Alloxan (50-71-5)	mouse	BM	Tollinger et al., 1974	8, 10
	mouse	spleen	Tollinger et al., 1974	8, 10
Arsenic (7440-38-2)	man	PBLC	Petres et al., 1977	1, 2
Azathioprine (446-86-6)	rabbit	PBLC	van Went, 1979	2
	man	PBLC	van Went, 1979	2
Benzene (71-43-2)	man	PBLC	Fredga et al., 1979	2 [d]
Benzo[*a*]pyrene (50-32-8)	rat	BM	Rees et al., 1979	2
2,7-bis(Acetylamino)fluorene (304-28-9)	rat	liver	Masashito et al., 1976	4
Bleomycin (11056-06-7)	rat	BM	Bishun et al., 1978	4, 10
1-Butylnitrosourea (869-01-2)	rat	liver	Masashito et al., 1976	4
Cadmium (7440-43-9)	man	PBLC	Bui et al., 1975	2
Cadmium sulfate (10124-36-4)	mouse	ascites tumor	Bishun and Pentecost, 1981	4, 10
Cannabinol (521-35-7)	man	PBLC	Matsuyama and Fu, 1981	2
Captan (133-06-2)	rat	BM	Shirasu et al., 1977	10
Carbon disulfide (75-15-0)	rat	BM	Vasil'eva, 1982	5
	rat	embryo	Vasil'eva, 1982	5
Cyclohexylamine HCl (4998-76-9)	C. hamster	PBLC	van Went-de Vries et al., 1975	2, 6
p,*p*'-DDT (50-29-3)	rat	liver	Masashito et al., 1976	2
Diethyl mercury (627-44-1)	man	PBLC	Verschaeve et al., 1976	2, 3
Diethylnitrosamine (55-18-5)	rat	liver and spleen	Grover and Fischer, 1971	4
	rat	liver	Masashito et al., 1976	4
Diethylstilbestrol (56-53-1)	mouse	ascites tumor	Bishun et al., 1980	3, 4
Diethylstilbestrol diphosphate (522-40-7)	mouse	embryo	Chrisman, 1974	8, 10
	mouse	BM	Christman and Hinkle, 1974	8, 10
	mouse	BM	Tollinger et al., 1974	8, 10
	mouse	spleen	Tollinger et al., 1974	8, 10
	mouse	BM	Ivett and Tice, 1981	8, 10
4-Dimethylaminoazobenzene (60-11-7)	rat	liver	Masashito et al., 1976	4

TABLE 2 (continued)

Chemical (CAS Registry No.)	Species [a]	Tissue [b]	Reference	Primary reason(s) for inconclusive [c]
Methyluracil (27942-00-3)	mouse	BM	Ill'inskikh, 1982	6, 10
Mitomycin C (50-07-7)	rat	BM	Teramoto et al., 1977	10
	rat	BM	Shirasu et al., 1977	6, 10
Morpholine (110-91-8)	S. hamster	embryo C	Inui et al., 1979a	3, 4
Nitrosomorpholine (59-89-2)	S. hamster	embryo C	Inui et al., 1979a	3, 4
Phenylmercuric acetate (62-38-4)	man	PBLC	Verschaeve et al., 1978	8, 10
Phenytoin (57-41-0)	rat	BM	Alving et al., 1976	6, 10
Phosphonoacetyl-L-aspartic acid (51321-79-0)	mouse	embryo C	Sieber et al., 1980	3, 4, 6, 10
Potassium dichromate (7778-50-9)	rat	BM	Bigaliev and Elemesova, 1978	4
	mouse	BM	Paschin et al., 1982	2
Progesterone (57-83-0)	rabbit	blastocyst	Widmeyer and Shaver, 1972	4, 5
β-Propiolactone (57-57-8)	rat	BM	Rees et al., 1979	2
Saccharinate sodium (128-44-9)	C. hamster	BM	van Went-de Vries and Kragten, 1975	2, 7
Sodium nitrate (7631-99-4)	S. hamster	embryo C	Inui et al., 1979b	4
Sodium nitrite (7632-00-0)	S. hamster	embryo C	Inui et al., 1979a	3, 4
	S. hamster	embryo C	Inui et al., 1979b	4
Styrene (100-42-5)	man	PBLC	Meretoja et al., 1977	2
	man	PBLC	Meretoja et al., 1978a	2
	rat	BM	Meretoja et al., 1978b	2
	man	PBLC	Meretoja and Vainio, 1979	2
	man	PBLC	Hogstedt et al., 1979	7
	man	PBLC	Watanabe et al., 1983	2
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (1746-01-6)	man	PBLC	Tenchini et al., 1979	3
	man	fetal fibro C	Tenchini et al., 1979	3
Δ-9-Tetrahydrocannabinol (1972-08-3)	rat	PBLC	Matsuyama and Fu, 1981	2
(−)-*trans*-Cannabidiol (13956-29-1)	man	PBLC	Matsuyama and Fu, 1981	2
Zinc oxide (1314-13-2)	rat	BM	Voroshilin et al., 1978	4

[a] Species: dog, beagle; C. hamster, Chinese hamster; S. hamster, Syrian golden hamster; monkey, Rhesus monkey.
[b] Tissue; BM, bone marrow; C, culture; fibro, fibroblast; PBLC, peripheral blood lymphocyte culture; (A), based on anaphase effects; (M), based on micronucleus frequency; (S), based on structural aberrations.
[c] Reasons: Numbers correspond to reasons given in Section 2.
[d] Sum of percentages exceeded 100%.
[e] Only tetraploidy reported.
[f] Some times of dose administration cause exposure of female germ cells.
[g] Deemed inconclusive due to different species used for different endpoints. See Section 6.

TABLE 2 (continued)

Chemical (CAS Registry No.)	Species [a]	Tissue [b]	Reference	Primary reason(s) for inconclusive [c]
Dimethylnitrosamine (62-75-9)	S. hamster	embryo C	Inui et al., 1979b	4
	C. hamster	liver	Swindell and Ockey, 1983	2
	C. hamster	liver C	Swindell and Ockey, 1983	2
Dimethyl sulfoxide (67-68-5)	rat	BM	Barilyak et al., 1978	6
2,4-Dinitrotoluene (121-14-2)	dog	BM C	Ellis et al., 1979	3 [e]
	dog	kidney	Ellis et al., 1979	3 [e]
	rat	BM C	Ellis et al., 1979	10
	rat	kidney	Ellis et al., 1979	10
Diphenylmercury (587-85-9)	man	PBLC	Verschaeve et al., 1976	2, 3
β-Estradiol dipropionate (113-38-2)	rabbit	blastocyst [f]	Widmeyer and Shaver, 1972	4, 5
Ethanol (64-17-5)	rat	BM	Barilyak and Kozachuk, 1980	9
	rat	fetus	Barilyak and Kozachuk, 1981	7, 8
	rat	BM	Barilyak and Kozachuk, 1981	7, 8
Ethylenethiourea (96-45-7)	rat	BM	Teramoto et al., 1977	10
	rat	BM	Shirasu et al., 1977	1, 10
Heroin (561-27-3)	monkey	PBLC	Fischman et al., 1983	9
	monkey	BM	Fischman et al., 1983	9
Lead (7439-92-1)	man	PBLC	Verschaeve et al., 1979a	8, 9
Lead nitrate (10099-74-8)	mouse	ascites	Bishun and Pentecost, 1981	4, 10
α-Lindane (319-84-6)	rat	liver	Masashito et al., 1976	4
Lysergic acid diethylamide (50-37-3)	C. hamster	BM	Moorthy and Mitra, 1978	2
	mouse	BM	Mitra et al., 1979	2
	mouse	spleen	Mitra et al., 1979	2
Maleic hydrazide (123-33-1)	rat	liver	Masashito et al., 1976	4
6-Mercaptopurine (50-44-2)	rat	BM	Barilyak et al., 1978	9
Mercuric chloride (7487-94-7)	S. hamster	BM	Watanabe et al., 1982	7
Mescaline (54-04-6)	C. hamster	BM	Moorthy and Mitra, 1979	2, 4
L-Methionine (63-68-3)	rat	liver	Masashito et al., 1976	4
Methyl-2-benzimidazole carbamate (10605-21-7)	mouse	BM (A)	Seiler, 1976	7 [g]
	mouse	BM (M)	Seiler, 1976	7 [g]
	C. hamster	BM (S)	Seiler, 1976	7 [g]
2-Methyl-4-dimethylaminoazobenzene (54-88-6)	rat	liver	Masashito et al., 1976	4
3'-methyl-4-dimethylaminoazobenzene (55-80-1)	rat	liver	Masashito et al., 1976	4
Methylmercuric chloride (115-09-3)	S. hamster	BM	Watanabe et al., 1982	7
Methylmercury (22967-92-6)	man	PBLC	Skerfving et al., 1974	2, 3

tion. Polyploidy, hypoploidy, and hyperploidy should be reported separately for assessment of their relative contributions to numerical deviations. Data should not be reported as percentages or frequencies unless the number of cells upon which they are based are given also. It is informative, but not absolutely necessary, for individual chromosome numbers to be tallied as a frequency distribution.

Statistically analysis should be performed. This is minimally a one-tailed (not a two-tailed) test. Nonparametric tests using individual subject (or animal) data appear to be the most appropriate methods at this time, but critical review of statistical tests for aneuploidy data is needed.

(4.1.8) Evidence for toxicity. Evidence that a dose level with toxic effects has been achieved is required for a definitive negative call to be made. In the absence of some indication of toxicity, chemicals thought to be negative will be subject to reassessment when examined at higher dose levels.

(4.2) Experimental mammal studies

(4.2.1) Choice of species. Mice are commonly used in this and other cytogenetic assays because there are genetically well-characterized strains and because mice are inexpensive and readily available. Other rodent species (e.g., rats, Chinese hamsters) or other mammalian species also can be used and in specific cases may be more appropriate. Comparative study of alternative species is encouraged.

(4.2.2) Number of animals. The number of animals needed depends upon the baseline frequency of aneuploidy in the species, strain and tissue, and upon the extent of inter-animal variability. Although somewhat arbitrary, 4 animals per sex per treatment group is considered a minimum, not necessarily an optimum, number. The use of small numbers of animals increases the chance of obtaining a false negative response.

(4.2.3) Sex differences. It is currently unknown if there are differences between the sexes in regard to mitotic aneuploidy induction. However, sex differences have been reported in responsiveness to some agents in the micronucleus assay (Henry et al., 1980) and in the sister-chromatid exchange assay (Tice et al., 1982). Unless it can be shown that no difference in sensitivity exists for aneuploidy induction, both males and females should be used in each treatment group.

(4.2.4) Cell population to be scored. Bone marrow cells have the advantages of being highly proliferative and easy to extract, and of having much correlative data for other chromosomal endpoints. Other tissues may be more appropriate as the target tissue for specific agents for pharmacokinetic reasons. Peripheral blood lymphocytes are convenient to obtain and would provide direct comparison with human studies (Section 4.1.6).

(4.2.5) Route of administration. The preferred route of administration is usually that by which humans are exposed to the same agent. For occupational or environmental exposures, these routes are oral, dermal and inhalation. The most common routes for therapeutic exposures are oral, intramuscular, or intravenous. The oral and intraperitoneal routes are used most commonly in short-term genetic toxicology assays.

(4.2.6) Dose levels and frequency. A dose producing some indication of toxicity is required to establish a creditable negative response. Toxicity is not necessary in studies resulting in a positive response. The maximum practical dose that can be administered (limited, for example, by solubility considerations) may be used for non-toxic compounds. Lower doses must be tested as well to assure that a negative response is not due to the preferential survival of a small, non-representative population of euploid cells. Cells severely damaged at high doses may not survive, while the less damaged cells from lower doses may survive long enough to be scored. Either acute (single) or multiple administrations of the chemical may be employed.

(4.2.7) Number of cells scored. A reasonable minimum number of cells to score depends upon the baseline frequency for controls. Hyperploid frequencies from the existing literature range from 0 to 4% (Tables 3 and 4), suggesting that it may be necessary to score a few hundred cells to avoid an appreciable number of zero-event animals (i.e., controls with no spontaneous hyperploid cells). Additional data are needed to establish baseline frequencies for the different species and strains of test animals and to determine reliably the minimum number of cells to be scored.

(4.2.8) Sampling time. Different chemicals may

from studies considered inconclusive (Table 2). Two additional chemical agents were excluded from Table 2 either as being of an ill-defined nature (mercury amalgam) or as being a mixture (organic mercury mixture). Other chemicals reported with these two had prevented these papers from being excluded during the initial screening. 12 of the chemicals in Table 2 involved human studies (data from 14 papers). One of these, styrene, was tested in both human and experimental animal studies. In animal studies, 51 other chemicals were described in 38 reports.

(4) Factors to be used in the development of a protocol

Although the acceptable data base is very limited, some recommendations can be made for studying aneuploidy induction using direct chromosomal counts in the somatic cells of mammals in vivo.

(4.1) Human studies

Many of the recommendations made by Galloway and Ivett (1985) for aneuploidy testing in mammalian cells in vitro apply for human cells in vivo. The major exceptions involve dose administration and metabolic activation; ethical considerations preclude experimental exposure of humans to possible aneuploidy-inducing agents, and exogenous metabolic activation is unnecessary during in vivo exposure.

(4.1.1) Controls. A concurrent control group should be employed, matched as closely as possible to the exposed group for age, sex, and life-style.

(4.1.2) Exposure levels. Because conditions and levels of exposure frequently are uncontrolled in human studies, methods for assessing the actual doses received by exposed individuals are essential. Analysis of metabolites in body fluids or of adducts in blood or of tissue proteins or nucleic acids can be performed for this purpose.

(4.1.3) Culture conditions. If cells must be cultured to obtain metaphases for analysis, established procedures should be used and culture conditions (e.g., pH and temperature) closely controlled.

(4.1.4) Number of subjects. The number of subjects needed for a proper evaluation depends upon the baseline frequency of aneuploidy. Use of a small number of subjects increases the chance of obtaining a false negative response.

(4.1.5) Number of cells scored. The number of cells scored depends on the baseline hyperploid frequency, which is expected to be low, about 0.5% (Section 5). The number of cells to be scored per individual is large, on the order of several hundred, to ensure detection of a biologically significant increase in hyperploid frequency.

(4.1.6) Cell population to be scored. Somatic cell aneuploidy in humans usually is measured in peripheral blood lymphocytes. Only first generation mitogen-stimulated metaphase cells should be scored if the objective is to detect aneuploidy induced only during lymphocyte production in the stem cell compartment. Damage to mature lymphocytes which could result in the production of aneuploid cells upon mitogenic stimulation can be detected in metaphase cells which have divided, not once, but two or more times. Aneuploid cells in second or third generation metaphase can be induced in whole blood cultures if aneuploidy-inducing chemicals are present in the serum. The proliferation kinetics of the human lymphocytes in culture is extremely complex; depending upon culture conditions and sampling time, metaphase cells which have divided one, two or more times since mitogenic stimulation can be obtained on a single slide. Discrimination between such cells can be made by bromodeoxyuridine differential staining.

Use of other somatic cell populations (e.g., bone marrow) is described for experimental animal studies (Section 4.2.4).

(4.1.7) Data accumulation and reporting. Slides should be coded prior to scoring to avoid reader bias. Only well-spread metaphases without apparent cell breakage should be scored. Chromosomes which from their staining characteristics or degree of condensation obviously do not belong to the subject metaphase should not be counted. The entire cell should be excluded from analysis before counting if uncertainty exists about its true chromosomal constitution. The exact chromosome number should be recorded for each cell scored, except in cases that are obviously polyploid.

Categories such as 'aneuploidy', 'numerical aberrations' or 'in excess of the diploid number of chromosomes' do not provide sufficient informa-

TABLE 4

EXAMPLES OF BASELINE FREQUENCIES OF HYPERPLOID CELLS IN BONE MARROW TISSUE IN MAMMALS

Species	Strain	Sex	Number of animals	Cells/ animal	Total cells	Solvent or vehicle	Hypo-ploid (%)	Hyper-ploid (%)	Aneu-ploid (%)	Reference
Chinese hamster		M and F	10	?	1900	Feed	9.47	0.21	9.68	Renner et al., 1982
Syrian golden hamster		F	5	50	250	Saline	2.6	0.6	3.2	Watanabe et al., 1982
		F (1 mo)	5	50	250	Untreated	1.2	0.4	1.6	Weiler and Sachsse, 1978
		F (10 mol)	15	50	750	Untreated	7.82	3.41	11.23	Weiler and Sachsse, 1978
		F (17 mo)	17	50	850	Untreated	13.64	3.31	16.95	Weiler and Sachsse, 1978
Rhesus monkey (*Macaca mulatta*)		F	4	100	400	Saline	1.92	0	1.92	Fischman et al., 1983
Mouse	Goodale	M	12	25	300	Saline	0–5.9	1.0–4.0	2.0–7.0	Tollinger et al., 1974
	LP/Jx SJL/Jx BALB/cJx C57BL/6J	M	20	25	500	Saline	0	0–1.6	0–1.6	Chrisman and Hinkle, 1974
Rat		M	10	?	1500	3:1 dH_2O: Tween 80	13.1	0.7	13.8	Barilyak et al., 1978
	Wistar	M	11	?	600	dH_2O	15.0	0.3	15.3	Barilyak and Koza-chuk, 1980
	Wistar	F	10	?	800	Sunflower seed oil(?)	11.4	0.4	11.8	Vasil'eva, 1982
	Wistar	M and F	4	100	400	dH_2O		0.2		Teramoto et al., 1977
	Wistar	M	6	50	300	Not indicated		0		Shirasu et al., 1977
	Wistar	F	10	?	?	dH_2O	12.9	0.7	13.6	Barilyak and Koza-chuk, 1981
	Lister	F	5	~ 50	~ 250	Not indicated		0.4		Alving et al., 1976
	CD	M and F	12	?	200	Feed	6	2	8	Ellis et al., 1979

cate a reported hyperploid frequency ranging from 0.1 to 4.17%, with a mode of about 0.4%, without regard to sex. There is an indication of an age dependence in aneuploidy frequencies (hypoploidy and hyperploidy) among humans. The data from experimental mammal (mostly rodent) studies (Table 4) indicate a hyperploid frequency ranging from 0 to 4.0%, with a modal frequency of 0.5% without regard for species, strain, sex, age, or nature of the solvent/vehicle control.

More detailed analysis of baseline frequencies will be necessary to establish the appropriate statistical modeling to be used in determining the capability of chemical agents to induce aneuploidy

induce aneuploidy by interacting with different parts of the mitotic process, occurring at different times during the cell cycle. Aneuploidy induction can be assessed properly and completely only by sampling at several intervals after treatment. Sufficient time must pass after treatment to permit expression of an aneuploidy event; the first post-treatment metaphase is not useful in this regard. Furthermore, the test agent may affect the rate of cellular proliferation, especially at high dose levels. For rodent bone marrow cells, 3 sampling times, at 24, 48, and 72 h after treatment, may be adequate for the majority of studies. Negative controls should be sampled at the same times as the treated animals.

(4.2.9) Data accumulation and reporting. Data accumulation and reporting should be the same as for human studies (Section 4.1.7).

(4.2.10) Recommended protocol. It is still too early in the development of this test to recommend a specific protocol. However, consideration of the above factors should provide guidance for the generation of data that will bear critical scrutiny and that can be used to support specific protocol recommendations.

(5) Evaluation of baseline frequencies for aneuploidy

Baseline (control) frequencies of aneuploidy have been collected from the literature in the EMCT data base, including some of the 112 papers initially rejected because they dealt with non-chemical agents. Only papers in which data on hyperploidy were separately reported were used; any accompanying data on hypoploidy and total aneuploidy was also extracted from these papers. No preconditions were applied to include or exclude frequencies of a given magnitude. The data presented in Tables 3 and 4 are not all-inconclusive, but they do provide some indication of the range of the baseline data reported in the literature. The data from human studies (Table 3) indi-

TABLE 3

EXAMPLES OF BASELINE FREQUENCIES OF HYPERPLOID CELLS IN PHYTOHEMAGGLUTININ-STIMULATED PERIPHERAL BLOOD LYMPHOCYTE CULTURES

Gender	Number	Age (years)	Cells/ person	Number of cells total	Termination time (h)	Hypoploid (%)	Hyperploid (%)	Aneuploid (%)	Reference
Male	10	34	100	1000	52	1.4	0.1	1.5	Popescu et al., 1979
	20	20–49	~100	1950	48	5.24	1.77	7.01	Verschaeve et al., 1979b
	8	19–25	100	800	43–48	3.33	0.11	3.44	Hirai, 1970
	9	65–74	100	900	43–48	6.38	1.88	8.26	Hirai, 1970
	12	24–63	50	600	48	15.50	4.17	19.67	Verschaeve et al., 1978, 1979a
Female	126		?	2164	72	4.95	0.40	5.35	Bishun et al., 1975
	114 [a]		?	2126	72	5.20	0.40	5.60	Bishun et al., 1975
Not reported	6	~21–56	200	1200	72		0.3		Hogstedt et al., 1979
	157	Babies	?	3686	72	5.30	0.20	5.50	Bishun et al., 1975
	153 [b]	Babies	?	3550	72	6.36	0.37	5.60	Bishun et al., 1975

[a] Used oral contraceptives.

[b] Were born to mothers who have used oral contraceptives.

and to determine the appropriate sample size for the numbers of animals and of cells to be evaluated. This is an important area for future investigation.

(6) Possible alternative methods for aneuploidy assessment for somatic mammalian cells in vivo

At the present time, actual numerical counting of chromosomes yields the only reliable evidence of aneuploidy induction by chemical compounds in somatic cells of mammals in vivo. After substantial improvement and validation, several other methods may provide alternative techniques in the future.

The analysis of anaphase configurations in cultured cells for multipolar spindles and lagging chromosomes has been used to indicate potential aneuploidy-inducing capability. Either of these two events should result in aneuploid cells. Two chemicals that reportedly induce such manifestations of aberrant disjunction in vivo are titanocene dichloride and vanadocene dichloride (Kopf-Maier, 1982). Inability of a chemical to induce multipolar spindles does not mean that the chemical cannot induce aneuploidy. Other mechanisms are possible for aneuploidy induction; thus, a lack of multipolar spindles, which are likely to be cell lethal, cannot be considered as definitive negative data. Lagging intact chromosomes can be confused with lagging acentric chromosome fragments, which are indicative of clastogenic damage; concurrent negative clastogenic data are required to support a claim for whole lagging chromosomes. Anaphase analysis is useful and informative, but ancillary information is required for proof of aneuploidy induction. Accumulation of mitoses after exposure to a chemical agent provides suggestive evidence for aneuploidy-inducing potential (Liang and Satya-Prakash, 1985). Once again, failure to produce this endpoint would not support a negative conclusion for aneuploidy induction.

Changes in nuclear DNA content can be detected readily by flow cytophotometry. Otto et al. (1981) found an increased coefficient of variation in mouse bone marrow cells exposed in vivo to cyclophosphamide. However, due to the large inherent variance in flow cytophotometric measurements, especially of cells treated with clastogenic agents, and because nuclear effects other than numerical aberrations alone might account for the above-discussed effects, this endpoint is not presently considered as a unique indicator of aneuploidy.

The possible significance of micronucleus size, with larger micronuclei presumably containing whole chromosomes and smaller ones containing fragments, was discussed by Yamamoto and Kikuchi (1981) as a method for distinguishing micronuclei caused by chromosomes from those caused by clastogenesis. Because small micronuclei may fuse to produce large micronuclei morphologically indistinguishable from those produced by aneuploidy induction, such analysis cannot stand alone. The use of micronuclei together with data on anaphase effects and on structural aberrations, as done by Seiler (1976; Section 2.7), is another potential method for obtaining information on aneuploidy induction.

Premature or delayed separation of centromeres during anaphase could lead to increased aneuploidy if cytokinesis occurs with chromatids unequally distributed (Miltenburger et al., 1980). Significant increases of C-mitosis, chromosome stickiness, and diplochromosomes indicate disturbances of spindle function that might produce subsequent gain or loss of chromosomes (Chatterjee et al., 1982).

Any of these alternative methods may prove to be useful for acquiring data on aneuploidy induction by chemical agents. Evaluation of their potential requires further development and validation of protocols. The impact of chemical agents on this important source of genetic damage in humans ultimately must be assessed in mammalian cells.

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